BIOCONVERSION OF CO_2 TO ETHANOL AND OTHER COMPOUNDS

Thomas M. Wahlund, Tyrrell Conway, and F. Robert Tabita
Department of Microbiology, The Ohio State University,
484 West 12th Avenue, Columbus, Ohio 43210-1292

Keywords: Microbial CO_2 fixation, recombinant DNA, ethanol

INTRODUCTION

The light-driven process of CO_2 fixation and its conversion into a myriad of larger organic molecules is a remarkably successful strategy employed by all plants. Likewise, there are numerous types of bacteria that are capable of extremely efficient CO_2 fixation, including those that use light-driven processes, as well as several species that are capable of using the energy obtained from chemical oxidations to fuel CO_2 reduction in the dark. However, nowhere in nature can an organism be found that has coupled CO_2 fixation to significant ethanol production. With the realization that fully one-third of the corn starch used for biological ethanol formation is lost through the production of CO_2 gas, it would be desirable to discover some way to use this CO_2 for the production of value-added chemicals or other products of interest. In this paper, we discuss recent experiments to couple the metabolism of CO_2 to the synthesis of ethanol using recombinant DNA technology. A chimeric bacterium was constructed so that CO_2 may be metabolized to ethanol.

RATIONALE AND EXPERIMENTAL APPROACHES

A logical strategy for genetically engineering microorganisms with the capacity for efficient ethanol production became possible when the genes that encode the key enzymes of ethanol biosynthesis from pyruvate were cloned from the bacterium *Zymomonas mobilis* (Conway *et al.*. 1987a; Conway *et al.*, 1987b). Indeed the *Z. mobilis* pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adh*) genes, when expressed in *Escherichia coli*, were found to enable this host to divert a significant amount of its normal metabolic profile to ethanol synthesis (Ingram and Conway, 1988). Since a common metabolic intermediate of virtually all central metabolic pathways is pyruvate, there is considerable interest to couple pyruvate formation to ethanol synthesis. Many autotrophic bacteria use the reductive pentose phosphate or Calvin-Benson-Bassham (CBB) pathway to convert CO_2 to organic matter. Since pyruvate is a key intermediate here as well, we considered the possibility that some of this pyruvate might be converted to ethanol in a CO_2-fixing chimeric bacterium overexpressing the *Z. mobilis pdc* and *adh* genes.

Much has been learned of the molecular biology and biochemistry of CO_2 fixation in microorganisms (Tabita, 1988; Tabita, 1995) and recently the potential for the regulated expression of foreign genes under autotrophic growth conditions in phototrophic bacteria has become established (Falcone and Tabita, 1991). Although much still needs to be learned about promoter structure and other aspects of gene expression in autotrophic bacteria, it was considered well worth the effort to determine whether it would be feasible to use existing technology to convert CO_2 into ethanol. The basic idea behind this approach is illustrated in Figure 1, where it is envisioned that recombinant *Rhodobacter* species would reduce CO_2 to ethanol under purely autotrophic (organic carbon-free) growth conditions. In this scheme, hydrogen is used as the ultimate reductant in the absence of oxygen under photosynthetic growth conditions and pyruvate (a product of the CBB pathway) is reduced to ethanol through the mediation of plasmid-encoded *pdc* and *adh* genes (Figure 2). Alternatively, one may take advantage of the ability of these organisms to fix CO_2 in the dark in the presence of oxygen, again in the absence of organic carbon. The versatility of these bacteria is further underlined by their ability to also use various organic compounds, instead of hydrogen, as electron donors.

RESULTS

The feasibility of using *Rhodobacter* and related organisms for foreign gene expression was first determined by constructing a promoter-plasmid vector capable of directing the synthesis of an easily assayed indicator enzyme, ß-galactosidase (encoded by the *lacZ* gene of *E. coli*). A variety of growth conditions, known to influence CO_2 fixation-related gene expression, was assessed in both *R. sphaeroides* and the related organism *R. capsulatus*. These conditions included anoxygenic growth under photoautotrophic (PA) conditions in a CO_2/H_2 atmosphere, aerobic chemoautotrophic (CLA) growth conditions in the dark in a $CO_2/H_2/O_2$ atmosphere, anoxygenic photoheterotrophic (PH) growth conditions in the light using organic carbon as an electron donor, and aerobic chemoheterotrophic (CH) growth conditions in the dark in the presence of organic carbon. In *R. sphaeroides*, maximum ß-galactosidase activity was obtained

in PA-grown cells, which reflected the high level of RubisCO (the major CO_2 fixation enzyme of the CBB pathway) associated with these growth conditions in this organism (Tabita, 1988; Tabita, 1995; Falcone and Tabita, 1996). The lowest level of ß-galactosidase was found in PH-grown cells, which also was reflected by the lower levels of RubisCO. Intermediate levels were obtained in CLA-cultured *R. sphaeroides* and to our surprise CH-dependent growth yielded fairly high levels of ß-galactosidase, while the level of RubisCO was at its expected lowest point. In *R. capsulatus*, the same promoter-*lacZ* construct showed a definitive and pronounced enhancement of ß-galactosidase activity under CLA-growth conditions, with much lower activity in PA-grown cells, and basically background levels in PH and CH-cultured *R. capsulatus*.

The results with the promoter-*lacZ* fusion-indicator constructs generally yielded much lower levels of ß-galactosidase than we might have expected based on previous studies with the same promoter directing transcription of foreign RubisCO genes (Falcone and Tabita, 1993). There are several reasons why *lacZ* may not have been as highly expressed as the genes encoding RubisCO with this vector, including factors related to the posttranscriptional processing of the message. Despite these results, we determined if it might be possible to use this vector to express the *pdc* and *adh* genes in *R. sphaeroides* and *R. capsulatus*, under the various growth conditions reported above. Basically, the same pattern of *adh* gene expression was obtained as above, namely the highest levels of alcohol dehydrogenase activity were obtained under PA and CLA growth conditions with *R. sphaeroides* and *R. capsulatus*, respectively. Again, with this vector the level of *adh* expression was not nearly as great as we had hoped based on prior RubisCO gene expression studies. The regulated expression of *adh* (and also *pdc*) did, however, encourage us to examine whether genetically engineered *R. sphaeroides* and *R. capsulatus* were capable of synthesizing ethanol under CO_2-fixation conditions. Both strains produced between 0.07 and 0.08 percent (w/v) ethanol in culture filtrates of photoautotrophic-grown cells. Furthermore, both alcohol dehydrogenase activity and the amount of ethanol synthesized could be significantly enhanced by the addition of xylose to the growth medium under PH growth conditions. Up to 0.023 percent ethanol (w/v) was obtained in *R. sphaeroides* and about 0.014 percent ethanol was produced by *R. capsulatus* cultures, with maximum levels reached at a considerably faster rate for *R. capsulatus*.

Another factor to be considered in any microbial process to produce ethanol is the tolerance of the host organism to ethanol. Each strain of *R. sphaeroides* and *R. capsulatus* employed in our study was sensitive to fairly low concentrations of ethanol. In Figure 3, the specific growth rate and the maximium extent of growth of each organism in a xylose-mineral salts medium under PH growth conditions was determined. As shown in this figure, higher concentrations of ethanol most affected the extent of growth and not the rate of growth. However, the data do show that in terms of the specific growth rate, *R. capsulatus* was considerably more resistant to ethanol. It must be kept in mind, however, that both strains of *R. capsulatus* and *R. sphaeroides* are laboratory strains that have not been otherwise altered. Thus, it may be possibile to modify the existing strains, and select for higher alcohol tolerance, as we have for temperature tolerance (Weaver and Tabita, 1983). Another alternative would be to isolate new strains.

DISCUSSION

These studies conclusively show that CO_2 may be converted to ethanol by two species of *Rhodobacter* under anoxygenic conditions in the light or under dark aerobic growth conditions. A promoter-vector molecule, which had been previously shown to be maximally effective under growth conditions which favored active CO_2 fixation, was adapted for these studies; e.g. in the absence of organic carbon. Although the overall level of *pdc* and *adh* gene expression was not as great as we had expected, the fact remains that CO_2 was converted to ethanol, both in the light and in the dark. Current studies are devoted to optimizing the position of the relevant genes within this vector and to stabilizing the *pdc* and *adh* transcript. In addition, other potential vectors have been developed which might prove even more suitable. Surprisingly, the use of xylose in photoheterophic cultures substantially enhanced ethanol production with our original vector, perhaps precluding the need for hydrogen as an electron donor for metabolism. Since xylose is a major constituent of corn starch, its use to stimulate plasmid-directed ethanol production in an organism with a highly active reductive pentose phosphate pathway may lead to a novel source of biologically synthesized ethanol. In this vein, it will be important to determine the total amount of ethanol derived from CO_2 and/or xylose carbon since these bacteria are known to oxidize organic carbon sources to CO_2 and refix and use this CO_2 as an electron acceptor (Tabita, 1995).

ACKNOWLEDGMENTS

This work was supported by a grant from the Consortium for Plant Biotechnology Research.

REFERENCES

1. Conway, T. Osman, Y.A., Konnan, J.I., Hoffman, E., and Ingram, L.O. (1987a) Promoter and nucleotide sequences of *Zymomonas mobilis* pyruvate decarboxylase. J. Bacteriol. 169, 949-954.
2. Conway, T., Sewall, G.W., Osman, Y.A., and Ingram, L.O. (1987b) Cloning and sequencing of the alcohol dehydrogenase II gene from *Zymomonas mobilis*. J. Bacteriol. 169, 2591-2597.
3. Ingram, L.O., and Conway, T. (1988) Expression of different levels of ethanologenic enzymes from *Zymomonas mobilis* in recombinant strains of *Escherichia coli* . Appl. Env. Microbiol. 54, 397-404.
4. Tabita, F.R. (1988) Molecular and cellular regulation of autotrophic carbon fixation in microorganisms. Microbiol. Rev. 53, 155-189.
5. Tabita, F.R. (1995) The biochemistry and metabolic regulation of carbon metabolism and CO_2 fixation in purple bacteria. *In*, Anoxygenic Photosynthetic Bacteria (R.E. Blankenship, M.T. Madigan, and C.E. Bauer, eds.) pp. 885-914, Kluwer Acad. Publ. The Netherlands.
6. Falcone, D.L., and Tabita, F.R. (1991) Expression of endogenous and foreign ribulose 1,5-bisphosphate caraboxylase/oxygenase (RubisCO) genes in a RubisCO deletion mutant of *Rhodobacter sphaeroides*. J. Bacteriol. 173, 2099-2108.
7. Falcone, D.L., and Tabita, F.R. (1993) Complementation analysis and regulation of CO_2 fixation gene expression in a ribulose 1,5-bisphosphate carboxylase-oxygenase deletion strain of *Rhodospirillum rubrum*. J. Bacteriol. 175, 5066-5077.
8. Weaver, K.E., and Tabita, F.R. (1983) Isolation and partial characterization of *Rhodopseudomonas sphaeroides* mutants defective in the regulation of ribulose bisphosphate carboxylase/oxygenase. J. Bacteriol. 156, 507-515.

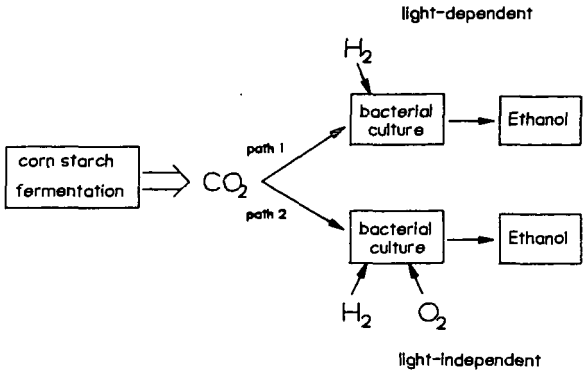

Figure 1. A general scheme to couple light-dependent and light-independent CO_2 fixation to ethanol production.

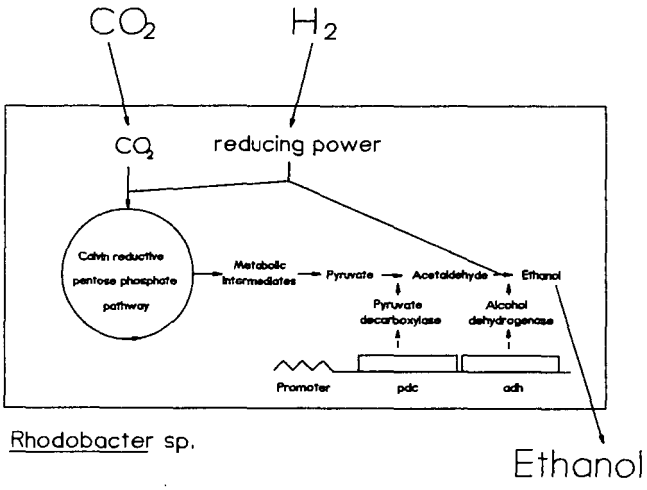

Figure 2. Ethanol synthesis using metabolic intermediates and pyruvate produced through the CBB reductive pentose phosphate pathway of CO_2 fixation.

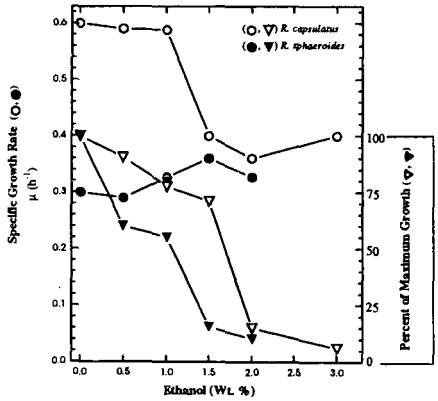

Figure 3. Specific growth rate (μ) and maximum extent (%) of growth in batch cultures of *R. sphaeroides* and *R. capsulatus* grown in a mineral salts-xylose medium containing different initial concentrations of ethanol. Cultures were bubbled with 1.5% CO_2/98.5% H_2 in the light.